

Gold Nanocomposite Bioink for Printing 3D Cardiac Constructs

Kai Zhu, Su Ryon Shin,* Tim van Kempen, Yi-Chen Li, Vidhya Ponraj, Amir Nasajpour, Serena Mandla, Ning Hu, Xiao Liu, Jeroen Leijten, Yi-Dong Lin, Mohammad Asif Hussain, Yu Shrike Zhang, Ali Tamayol, and Ali Khademhosseini*

Bioprinting is the most convenient microfabrication method to create biomimetic three-dimensional (3D) cardiac tissue constructs, that can be used to regenerate damaged tissue and provide platforms for drug screening. However, existing bioinks, which are usually composed of polymeric biomaterials, are poorly conductive and delay efficient electrical coupling between adjacent cardiac cells. To solve this problem, a gold nanorod (GNR)-incorporated gelatin methacryloyl (GelMA)-based bioink is developed for printing 3D functional cardiac tissue constructs. The GNR concentration is adjusted to create a proper microenvironment for the spreading and organization of cardiac cells. At optimized concentrations of GNR, the nanocomposite bioink has a low viscosity, similar to pristine inks, which allows for the easy integration of cells at high densities. As a result, rapid deposition of cell-laden fibers at a high resolution is possible, while reducing shear stress on the encapsulated cells. In the printed GNR constructs, cardiac cells show improved cell adhesion and organization when compared to the constructs without GNRs. Furthermore, the incorporated GNRs bridge the electrically resistant pore walls of polymers, improve the cell-to-cell coupling, and promote synchronized contraction of the bioprinted constructs. Given its advantageous properties, this gold nanocomposite bioink may find wide application in cardiac tissue engineering.

1. Introduction

Three-dimensional (3D) bioprinting is making strides in tissue engineering due to its precise deposition and patterning abilities of biomaterials. This technology has the ability to create highly organized 3D tissue constructs that mimic in vivo biologic conditions and can pave the way for organ transplantation, preclinical drug testing, and tissue morphogenesis studies.^[1] Previously, bioprinting was primarily used to fabricate acellular constructs, which were then seeded with cells for functionality. However, the efficiency of this strategy is limited due to the long distance of cell infiltrating and uncontrollable cell adhesion. On the contrary, cell-laden printing is a fabrication method, in which cells are premixed with biomaterials, and then deposited into desired patterns. This technology allows for easy

Dr. K. Zhu, Dr. S. R. Shin, T. van Kempen, Dr. Y.-C. Li, V. Ponraj, A. Nasajpour, S. Mandla, Dr. N. Hu, Dr. X. Liu, Dr. J. Leijten, Dr. Y. S. Zhang, Dr. A. Tamayol, Prof. A. Khademhosseini
Biomaterials Innovation Research Center
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
Cambridge, MA 02139, USA
E-mail: SSHIN4@partners.org; alik@mit.edu, alik@bwh.harvard.edu

Dr. K. Zhu, Dr. S. R. Shin, T. van Kempen, Dr. Y.-C. Li, V. Ponraj, A. Nasajpour, S. Mandla, Dr. N. Hu, Dr. X. Liu, Dr. J. Leijten, Dr. Y. S. Zhang, Dr. A. Tamayol, Prof. A. Khademhosseini
Harvard-MIT Division of Health Sciences and Technology
Massachusetts Institute of Technology
Cambridge, MA 02139, USA

Dr. K. Zhu
Department of Cardiac Surgery
Zhongshan Hospital
Fudan University
Shanghai 200032, China

Dr. S. R. Shin, Dr. Y.-C. Li, S. Mandla, Dr. Y. S. Zhang, Dr. A. Tamayol, Prof. A. Khademhosseini
Wyss Institute for Biologically Inspired Engineering
Harvard University
Boston, MA 02115, USA

Dr. J. Leijten
Department of Developmental BioEngineering
University of Twente
Enschede, Overijssel 7522 NB, The Netherlands

Dr. Y.-D. Lin
Divisions of Genetics and Cardiovascular Medicine
Department of Medicine
Brigham and Women's Hospital
Harvard Medical School
Boston, MA 02115, USA

Prof. M. A. Hussain
Department of Electrical and Computer Engineering
King Abdulaziz University
Jeddah 21569, Saudi Arabia

Prof. A. Khademhosseini
Department of Bioindustrial Technologies
College of Animal Bioscience and Technology
Konkuk University
Seoul 143-701, Republic of Korea

Prof. A. Khademhosseini
Department of Physics
King Abdulaziz University
Jeddah 21569, Saudi Arabia



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control of cell density and viability on the printed constructs and is thus considered potentially useful fabrication strategy for tissue engineering.^[2] An ideal cell-laden bioink should allow for the fluent extrusion of cells from the nozzle during printing without causing cellular damage through shear stress, or a decrease in the printing speed and therefore the resolution.^[3] It is also important to ensure that the bioink is cytocompatible and supports cellular growth and function by mimicking the microstructure architecture of native extracellular matrix (ECM).^[4] Furthermore, there is a need for tunable mechanical characteristics, which support the stability of the structure's architecture while maintaining cellular behaviors such as proliferation, spreading, and viability.

In our previous study, we developed a blend of gelatin methacryloyl (GelMA) and alginate prepolymers as a bioink for the encapsulation of endothelial cells.^[4,5] GelMA is a highly bioactive biomaterial due to the presence of integrin-binding motifs and matrix metalloproteinase-sensitive groups.^[6] To avoid physical crosslinking of the GelMA chains, alginate is used to maintain the viscosity of the bioink for prolonged periods of time while at room temperature.^[4] Using a coaxial extrusion needle system, a microfiber was printed and gelled through ionic crosslinking with calcium chloride (CaCl₂). Following bioprinting, the cell-laden microfibers were exposed to ultraviolet (UV) light to covalently crosslink the GelMA chains. A chemically stable and microporous GelMA microstructure was formed with biocompatibility for cell growth.^[6] As a result, a blend of GelMA and alginate prepolymers appears to be a useful bioink for cell-laden 3D bioprinting.

It has been shown that electrically conductive nanomaterials such as carbon nanotubes, graphene oxide, and gold nanoparticles could induce cardiomyocyte (CM) maturation and organization in 3D cardiac tissue constructs.^[7] Amongst the various electrically conductive nanomaterials, gold nanostructures, including nanospheres, nanorods, and nanowires, are considered as promising candidates in biomedical research, due to their biocompatibility, facile fabrication, and modification processes, and diverse aspect ratios.^[8] Notably, the conductive properties of gold nanostructures have been utilized to improve the electrical propagation between adjacent CMs.^[7c,9] In a recent study, a gold nanorod (GNR)-incorporated GelMA hydrogel patch was fabricated and subsequently seeded with CMs.^[9c] The CMs interacted with the GNRs and the engineered cardiac patch with GNR-GelMA hydrogel exhibited significantly faster and synchronous electrical signal propagation, as compared to that of pristine GelMA hydrogel constructs. Despite the advantages of such two-dimensional (2D) cardiac patch, a more convenient and efficient strategy to fabricate biomimetic 3D tissue is needed for enabling a range of applications to regenerate damaged tissue and provide platforms for drug screening. Bioprinting provides the possibility of the efficient fabrication. However, current hydrogel matrices used in cell-laden bioinks are electrically insulated, resulting in a delay in electrical propagation between adjacent cardiac cells within the constructs.^[2,10] Therefore, an electrically conductive component should be incorporated into such matrices to create a relevant bioink in order to bioprint 3D functional cardiac tissue constructs.

Cardiac fibroblasts (CFs) are predominant nonmyocardial cells in the native myocardium and are central in the regulation

of the ECM.^[11] Moreover, they are necessary for electrical propagation across the entire heart and have an effect on the phenotype of CMs through interactive communication.^[12] Many studies have exploited the coculture of CMs and CFs in engineered tissue constructs.^[12,13] However, the CFs in the construct tend to be highly proliferative during in vitro cultures, and as a result, they may impede the contraction of the entire engineered construct.^[9a] Interestingly, gold nanostructures were found to attenuate the overproliferation of CFs and maintain a suitable ratio of cocultured CMs and CFs in the constructs.^[9a,14] Therefore, the strategy of adding gold nanostructures is attractive for engineering cardiac tissue constructs. In this work, we developed a gold nanocomposite bioink by incorporating GNRs into a CM- and CF-laden hydrogel. We hypothesized that the GNR bioink-bioprinted cardiac constructs could provide a cytocompatible platform for the long-term survival and function of cardiac cells.

2. Results and Discussion

2.1. Evaluation of G-GNR/GelMA Hydrogel

Commercial cetyltrimethylammonium bromide (CTAB)-coated GNRs (C-GNRs, 34 nm long and 25 nm wide) were typically produced by a seed-mediated, surfactant-assisted approach, in which CTAB served as a tightly packed bilayer and cationic surfactant. This CTAB coating layer on the nanoparticles serves to maintain the colloidal stability and preserve the passivity of the particles' surfaces. Once the CTAB was removed from the surface, the gold nanoparticles aggregate, due to an attractive interparticle force.^[8b] However, the presence of CTAB on the GNR surface is incompatible with many biological systems due to its toxic nature to cells and tissues.^[15] Many strategies have been applied to tune the surface chemistry of C-GNR to reduce toxicity. One of the most popular method is layer-by-layer assembly technique, where additional layers of zwitterionic molecules are adsorbed onto the positively charged CTAB bilayer.^[16] Previously, a number of additional coating molecules, such as polystyrene sulfonate and poly(diallyldimethyl ammonium chloride), were used to coat CTAB layer of GNRs via electrostatic interactions.^[14b,17] As a result, the surface-coated GNRs showed good biocompatibility while maintaining the colloidal stability.

In this study, C-GNR was centrifugated repeatedly to remove excess CTAB in the stock solution. Afterward, GelMA molecules were coated on the surface of CTAB bilayer under ultrasonication. As a result, GelMA-coated GNRs (G-GNRs) could be redispersed homogeneously in aqueous solutions (Figure 1a). When gold nanostructures interact with light, different visible colors can result from their environment as well as size and structure.^[18] In our study, C-GNRs solution showed a ruby red color at a concentration of 0.1 mg mL⁻¹ (Figure 1a). After coating with GelMA molecules, the resultant G-GNRs solution showed a similar color to the original C-GNRs. Transmission electron microscopy (TEM) confirmed that the dense GelMA-coating layer did not significantly change the particle size as compared with the C-GNRs (Figure 1a). The UV-visible (UV-vis) spectral analysis revealed the presence of two

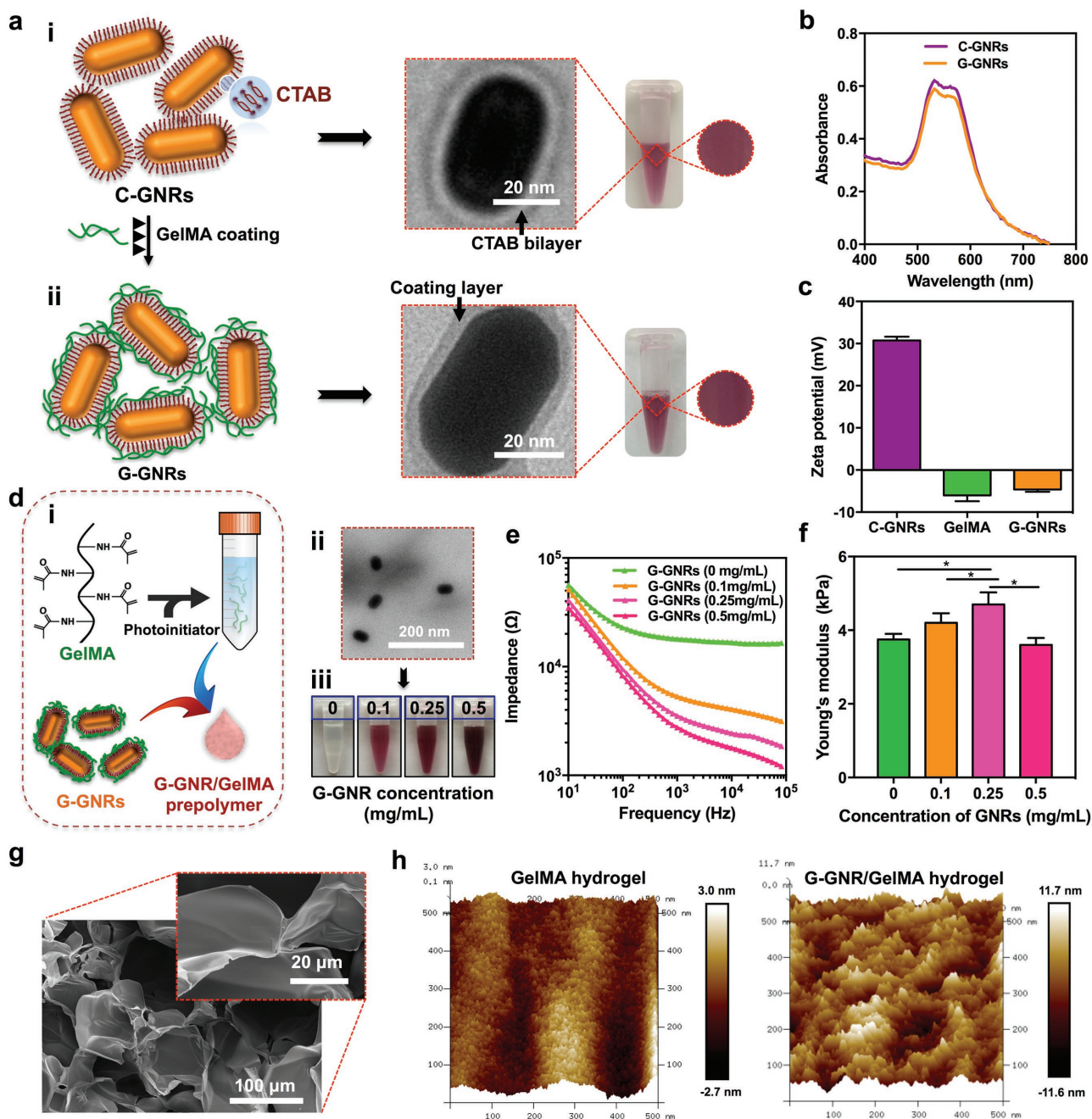


Figure 1. Preparation of G-GNR/GelMA hydrogel. a) Schematic of coating GelMA molecules on GNRs. i) CTAB bilayer was found on GNRs by TEM before treatment. ii) GelMA-coated layer was found on GNRs by TEM after treatment. b) UV-vis spectra showed G-GNRs were the same as that of C-GNRs, thus indicating that G-GNRs were prepared without changing aspect ratio and forming aggregates. c) Zeta potential of C-GNRs and G-GNRs. d) Schematic of preparation for G-GNR/GelMA prepolymer solution with various concentrations of G-GNRs. TEM image showed the uniform distribution of G-GNRs in the solution. Darker color was observed with higher concentrations of G-GNRs in the solution. e) Impedance and f) Young's modulus of G-GNR/GelMA hydrogel with various concentrations of G-GNRs. g) No obvious G-GNR aggregation was observed on the G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel matrix wall under SEM. h) AFM images of the pristine GelMA (7%) hydrogel and G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel.

surface plasmon bands; one at 520 nm and another at 600 nm. These peaks are indicative of solutions containing nanorods and correspond to the transverse and longitudinal oscillation modes, respectively. The absorption spectrum of G-GNRs was identical to that of C-GNRs, indicating that G-GNRs were prepared without changing the aspect ratio or forming aggregates

(Figure 1b). The zeta potential of C-GNRs and GelMA prepolymer solution were observed to be 30.73 ± 0.52 and -6.02 ± 0.80 mV, respectively (Figure 1c). Therefore, we can confirm that GelMA molecules were successfully coated on the GNRs as they had an anionic surface (-4.62 ± 0.33 mV) (Figure 1c). Furthermore, G-GNRs showed low cytotoxicity as compared

with C-GNRs, which indicated the G-GNRs could be a potential candidate for biomedical applications (Figure S1, Supporting Information).

The G-GNR/GelMA prepolymer solution was prepared by incorporating G-GNRs at various concentrations (from 0 to 0.5 mg mL⁻¹) into a 7% GelMA prepolymer solution (Figure 1d(i)). A photoinitiator was added to enable covalently crosslinking ability of the prepolymer solution. TEM image showed that the G-GNRs were homogeneously dispersed in the solution (Figure 1d(ii)). As expected, the G-GNR/GelMA prepolymer solution appeared darker in color with the increase of particle concentration (Figure 1d(iii)). The impedance was found to be reduced when more G-GNRs were present in the hydrogel (Figure 1e). Even at a low particle concentration (0.1 mg mL⁻¹), the impedance of the G-GNR/GelMA hydrogel was still significantly lower than that of the pristine GelMA hydrogel. Moreover, incorporation of G-GNRs increased the Young's modulus of the hydrogel following UV-mediated crosslinking (Figure 1f). However, we found that a high concentration (0.5 mg mL⁻¹) of G-GNRs in the GelMA hydrogel may result in an increased UV light reflection, which interfered with the crosslinking process, thus rendering softer gelation. A previous study reported that a cardiac tissue engineered with poly(2-hydroxyethyl methacrylate-co-methacrylic acid) exhibited a Young's modulus of 12 ± 6 kPa.^[19] In another study, 3D cardiac constructs composed of gold nanowires-incorporated alginate hydrogel exhibited a Young's modulus of 3.5 ± 0.2 kPa.^[7c] Similarly, 0.1 mg mL⁻¹ G-GNR/GelMA hydrogel was measured to have a low modulus (4.2 ± 0.3 kPa) following UV crosslinking. However, it has been reported that native neonatal rat myocardium and adult rat heart tissue have local moduli of 4.0–11.4 kPa (with a mean value of 6.8 kPa) and 11.9–46.2 kPa (with a mean value of 25.6 kPa), respectively.^[20] Native human myocardium has a measured modulus of 425 ± 9 kPa.^[7c] Despite the significantly lower modulus of the G-GNR hydrogel with respect to native adult heart tissues, we speculate that the artificial construct, with the low modulus, could benefit the encapsulated cardiac cells by promoting their organized contraction in the engineered cardiac tissues. Eventually with time, the construct will mature and more closely resemble the mechanical properties of native tissue as ECM is produced to replace the degrading biomaterial.^[7c,19] Scanning electron microscopy (SEM) revealed no obvious particle aggregation in the G-GNR/GelMA hydrogel (Figure 1g). At the nanoscale, atomic force microscopy (AFM) measured the surface topography of the trapped G-GNRs in the G-GNR/GelMA hydrogel (Figure 1h). As a comparison with pristine GelMA hydrogel, the G-GNR/GelMA hydrogel showed a relatively rough surface topography. We postulate that the raised surface of the G-GNRs could make contact with cell membranes for electrical propagation when cardiac cells are grown inside of the matrices.

To optimize the concentration of G-GNR and evaluate the CM behavior on the GNRs/GelMA hydrogel, hydrogel films (thickness = 50 μm) were fabricated as previously reported (Figure 2a).^[7b] CMs were seeded and then cultured on the film at various concentrations of G-GNRs. As a result, G-GNR/GelMA hydrogel was found to be superior when compared to the pristine GelMA hydrogel in terms of CM adhesion and retention ($P < 0.05$) (Figure 2b,c). Neonatal CMs can generate

the highest cellular force on materials, which have an elastic modulus in close proximity to that of the native neonatal rat myocardium (≈6.8 kPa).^[20,21] Consistently, our study found that the modulus of the G-GNR/GelMA hydrogel (0.1 and 0.25 mg mL⁻¹) was 4.2 ± 0.3 kPa and 4.7 ± 0.3 kPa, which is higher than the pristine GelMA hydrogel (3.75 ± 0.15 kPa), thus indicating a higher cell force and retention on the G-GNR/GelMA hydrogel. Although there was no meaningful difference in cellular retention at low concentrations of G-GNRs (0.1 and 0.25 mg mL⁻¹), high concentration of G-GNRs (0.5 mg mL⁻¹) rendered significantly less cell retention. This can be attributed to the soft substrate property (3.6 ± 0.19 kPa) as a result of the incomplete covalent crosslinking in the hydrogel (Figure 1f). Moreover, CMs on the G-GNR/GelMA hydrogel were characterized by immunostaining for F-actin (Figure 2d), as well as sarcomeric α-actinin and the gap junction protein connexin 43 (Cx-43) (Figure 2e), which are associated with the z-discs that define muscle sarcomeres and electrical coupling, respectively.^[7b] Elevated Cx-43 expression was observed on the G-GNR/GelMA hydrogel when compared to the pristine GelMA hydrogel. The constructs were also immunostained for troponin I, which is involved in muscle calcium binding and the contraction of CMs.^[7b] More troponin I expression was found within CMs cultured on the G-GNR/GelMA hydrogel than those on the pristine GelMA hydrogel (Figure 2f). As a result, CMs on the G-GNR/GelMA hydrogel started to show synchronous beating on day 2, and beat as an intact construct with a higher frequency by day 5. In comparison, CMs cultured on the pristine GelMA hydrogel did not show synchronous beating until day 5, which was at a slower rate than the G-GNR/GelMA hydrogels (Videos S1–S4, Supporting Information). Overall, G-GNR/GelMA hydrogel provided a beneficial micro-environment for CM retention, growth, and function.

2.2. Bioprinting Using Gold Nanocomposite Bioink

The gold nanocomposite bioink was composed with a blend of G-GNR-incorporated GelMA and alginate prepolymer solutions, thus allowing it to be universally used in various 3D bioprinting systems. The first example is a coaxial extrusion needle system (Figure S2 and Video S5, Supporting Information), which was reported previously.^[4] Bioink and calcium chloride (CaCl₂) were coextruded through the internal and external needles, respectively. When CaCl₂ came into contact with the bioink, the alginate prepolymer within the bioink was ionically crosslinked and resulted in hydrogel microfibers. The printability of gold nanocomposite bioink (0.1 mg mL⁻¹ G-GNRs in 7% GelMA prepolymer) was also evaluated while using different concentrations of alginate and CaCl₂ (Figure S3, Supporting Information). To minimize cell death in high CaCl₂ concentrations, we chose 2% alginate and 0.3 M CaCl₂ as the printing conditions. After layer-by-layer bioprinting of microfiber constructs, the GelMA chains were covalently crosslinked through UV exposure (Figure 3a). Using this system, G-GNR nanocomposite bioink was successfully applied to print layer-stacked constructs with different inner grid sizes (Figure 3b–d).

The continued presence of the red colored G-GNRs in the bioprinted constructs was visually followed during the entire

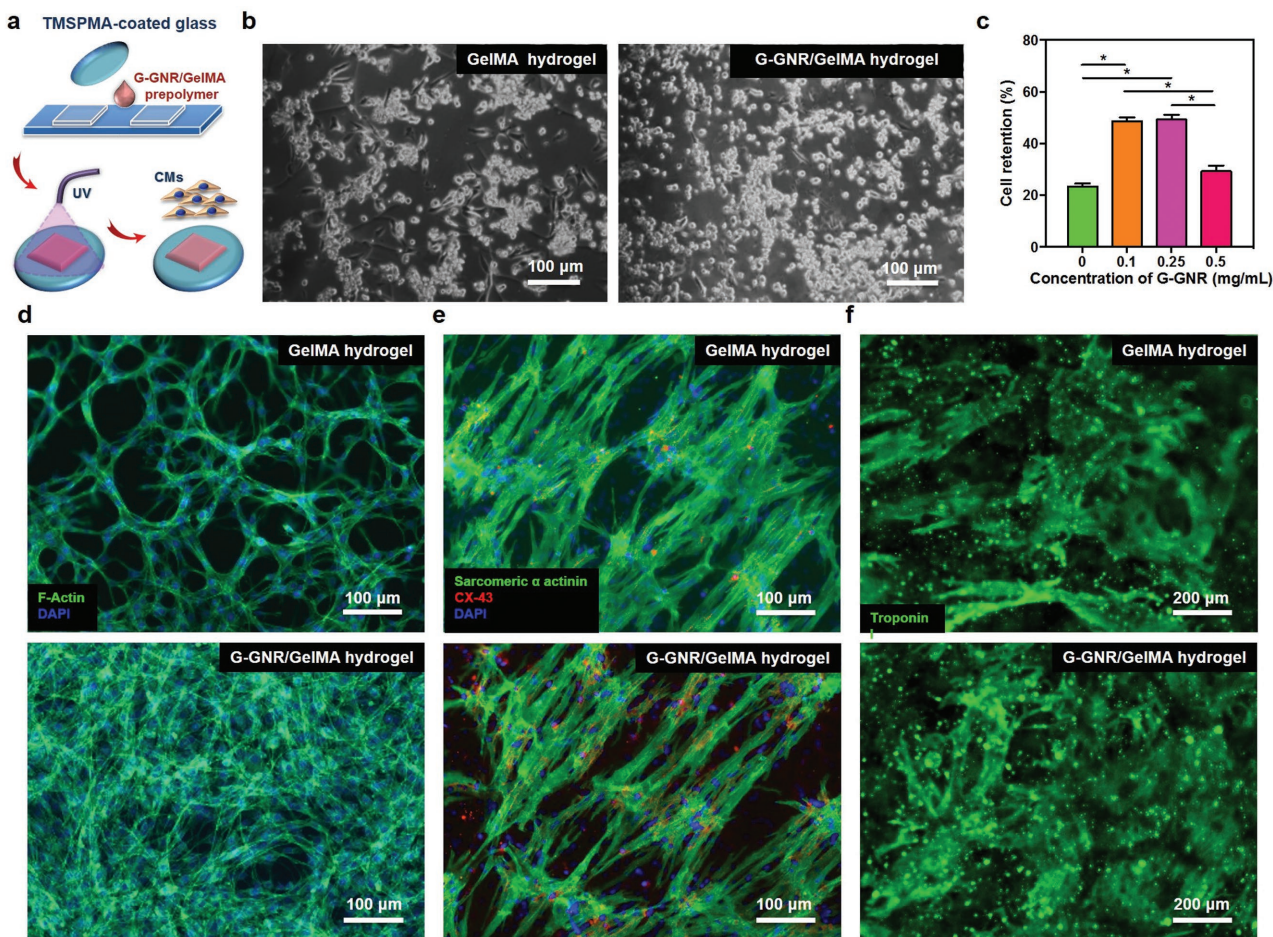


Figure 2. CMs on G-GNR/GelMA hydrogel. a) Schematic of G-GNR/GelMA hydrogel constructs. b) Optical images of CMs grown on the pristine GelMA (7%) hydrogel and G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel at day 1. c) Quantification analysis showed that CMs on the G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel had a better retention rate than those on pristine GelMA (7%) hydrogel at day 1 (* $P < 0.05$). d) The G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel showed a more uniform cell coverage on day 5 after cell seeding. e) Expression of sarcomeric α -actinin, Cx-43 and f) troponin I in CMs grown on pristine GelMA (7%) hydrogel and G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel at day 7.

culture period with the use of phenol red-free culture medium. No visible color change of the medium was observed during 5 d of culture at 37 °C. Following degradation of the construct with a collagenase type II solution at 37 °C, the colorless medium began to take on a red hue due to the release of the G-GNRs (Figure 3e). Absorbance spectrum analysis of the media also confirmed that the G-GNRs were retained in the constructs and only released upon forced degradation (Figure 3f). We assume that electrostatic interaction between the CTAB and GelMA as well as the covalently crosslinked GelMA network entrapped G-GNRs and prevented them from being released.

To obtain spatially complicated constructs, the printing process can be conducted in a hydrogel support bath system.^[22] In the present study, our G-GNR nanocomposite bioink was successfully printed as a spiral construct in a hydrogel support bath that was composed of 11×10^{-3} M CaCl₂ and 2% gelatin solution (Figure 3g). Rheology analysis showed that the gelatin support bath acted as a Bingham plastic at 4 °C (Figure S4, Supporting Information). This means that the bath can behave as a rigid body at low shear stresses, but flow as a viscous fluid at higher shear stresses. Thus the nozzle suffered little

mechanical resistance while moving through the bath, and the bioink was extruded out of the nozzle and remained in place within the support bath, yielding the intended spatially 3D geometry (Figure 3h). After UV light exposure, the bioprinted construct was obtained without damage by melting the gelatin support bath at 37 °C (Figure 3i).

2.3. Cellular Survival in the Bioprinted Constructs

A satisfactory cell viability is central to the cell-laden bioprinting technique. To improve cell viability during fabrication, we optimized multiple parameters, such as shear stress during printing procedure and UV exposure time to crosslink the printed cell-laden construct. In this study, CFs (5×10^6 cells mL⁻¹) were loaded into the G-GNR nanocomposite bioink for evaluating cell viability. The pristine GelMA/alginate bioink, which lacked G-GNRs, was used as a control in this study. Shear stress is inevitable when extruding a cell-laden bioink through a printer nozzle, and thus it plays a role in determining the cell viability after bioprinting (Figure 4a). The rheology of the bioink

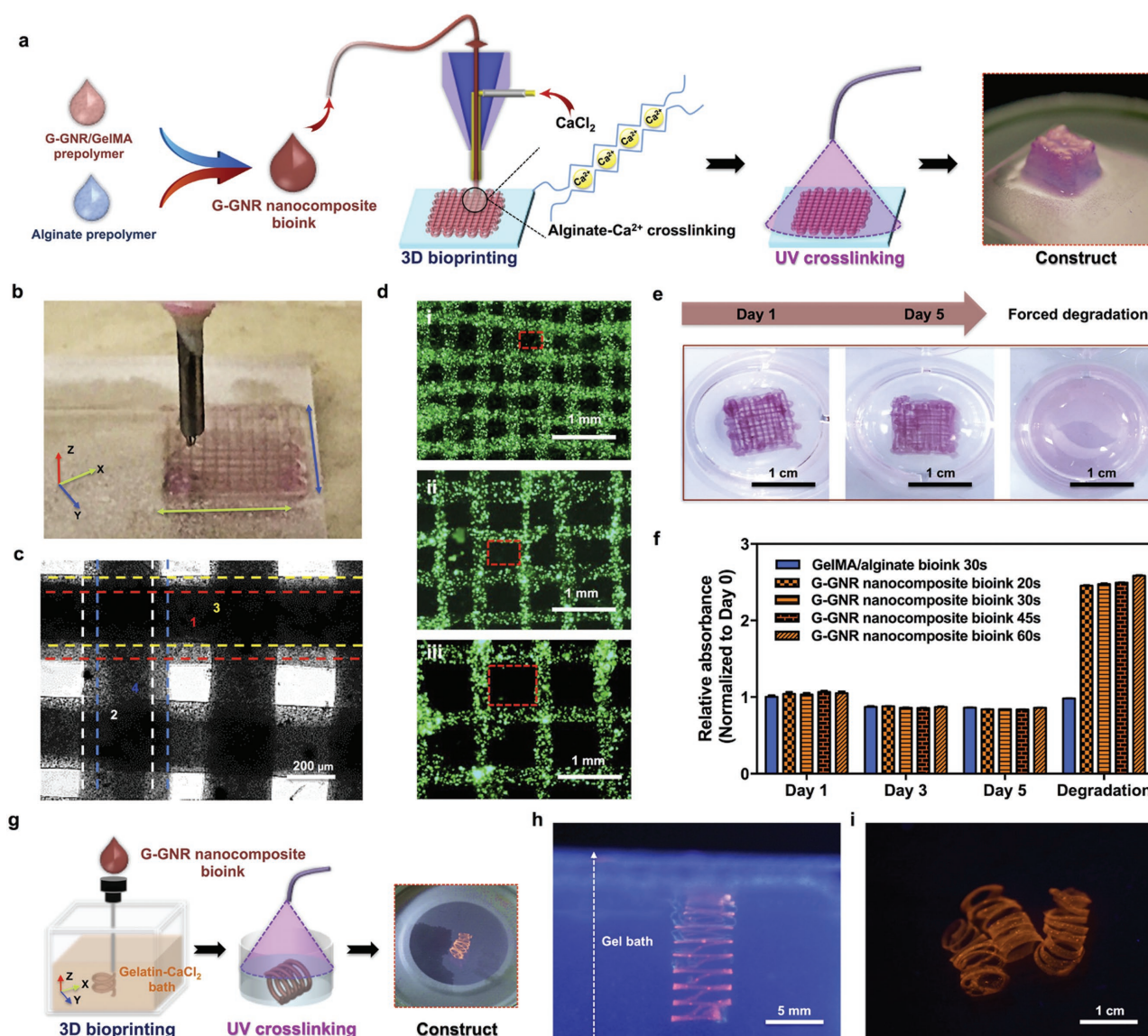


Figure 3. 3D bioprinting using G-GNR nanocomposite bioink. a) Schematic of bioprinting process using the G-GNR nanocomposite bioink. The inset showed the bioprinted 30-layered construct. b) The printing procedure on x, y, and z axes. c) Stacked layers (layers 1–4) using G-GNR nanocomposite bioink could be observed under microscope. d) Constructs with different inner grid could be bioprinted (green beads were embedded in G-GNR nanocomposite bioink to exhibit the printed fibers). e) Stable bioprinted constructs in culture medium up to day 5 and forced degradation of bioprinted construct using a collagenase solution at 37 °C. f) The absorbance spectrum analysis of culture medium confirmed that the G-GNRs were kept into bioprinted constructs and released upon forced degradation of constructs. g) Schematic of 3D embedded bioprinting using the G-GNR nanocomposite bioink. The inset showed the printed spiral structure in a support bath (2% gelatin, 11×10^{-3} M CaCl₂). h) The spiral construct in the support bath after printing. i) Bioprinted constructs could be obtained without damage and cultured in medium.

significantly influenced the amount of shear stress experienced when it was extruded from the nozzle.^[3] In our previous study, pristine GelMA/alginate bioink was extruded through the same nozzle system with a high cell viability (>70%).^[4] It has been well acknowledged that nanocomposite hydrogels exhibit different rheological properties when compared to their pristine hydrogel counterparts.^[23] Hence, it is important to explore the potential changes of the rheological properties upon G-GNR incorporation and their corresponding influence on cells. In the current study, we measured the viscosity of the G-GNR nanocomposite bioink (Figure 4b) and analyzed the velocity

profile of the bioink in the nozzle (Diameter = 200 μ m) using computational fluid dynamics (Figure 4c). We found that the G-GNR nanocomposite bioink exhibited typical power-law behaviors as seen in Equation (1). When compared to the pristine GelMA/alginate bioink, the presence of G-GNRs showed an increased flow consistency index (K) and a decreased flow behavior index (n) (Table S1, Supporting Information). This led to an amplified shear-thinning effect and improved printability, which resulted in a lower velocity in the nozzle. Based on Equations (1) and (2),^[3] we were able to calculate the shear stress distribution occurring inside the nozzle analytically.

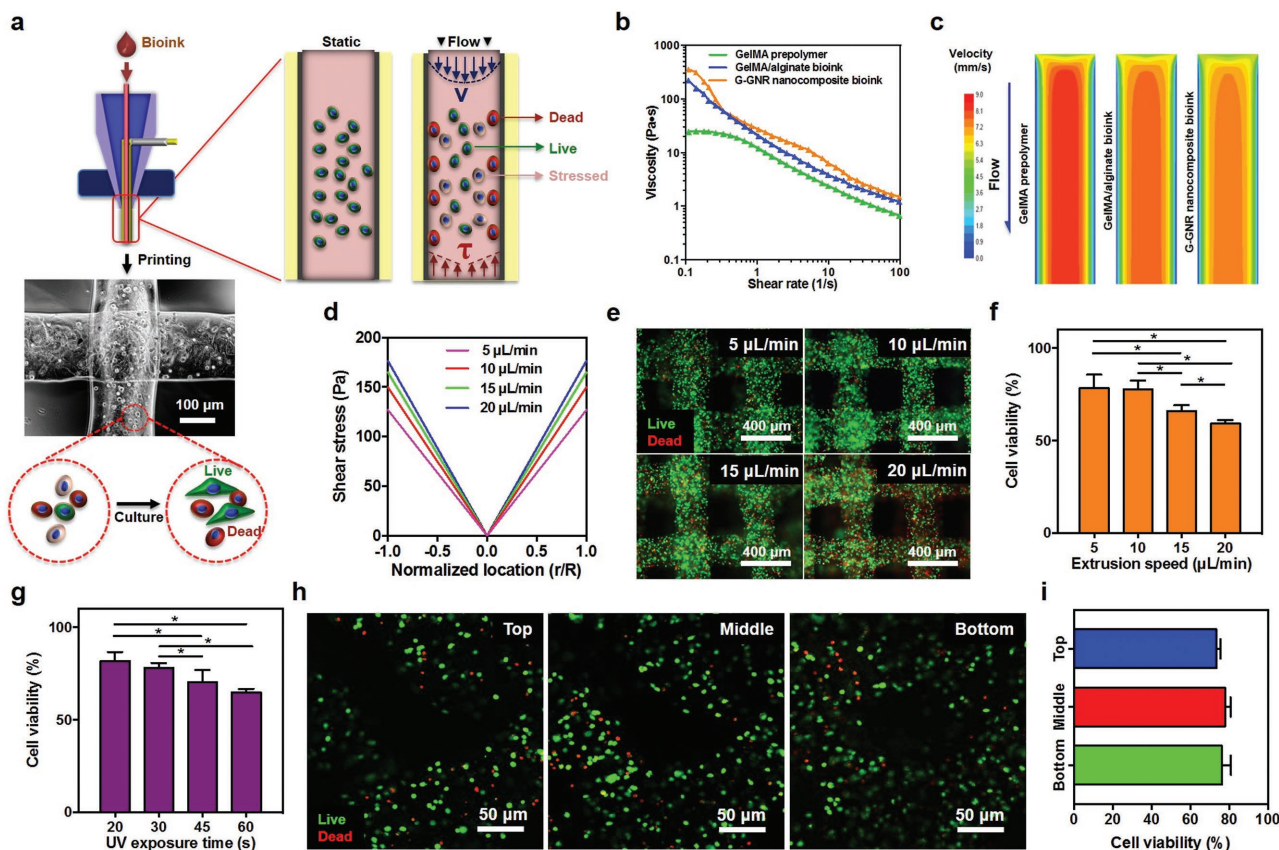


Figure 4. Cell viability in bioprinted constructs. a) Schematic of shear stress effect on cells inside of the printing nozzle. b) Rheological characterization of 7% GelMA prepolymer solution, GelMA/alginate bioink (7% GelMA, 2% alginate), and G-GNR nanocomposite bioink (0.1 mg mL⁻¹ G-GNR, 7% GelMA, 2% alginate). c) Computational simulation of velocity of the bioink in the nozzle while using the extrusion speed of 10 µL min⁻¹. d) The shear stress profile of the G-GNR nanocomposite bioink was higher with the increase of extrusion speed. r is the radial coordinate, where the origin is located at the axis of the printing needle. R is the radius of the printing needle. e, f) Live/dead assay of CFs within G-GNR nanocomposite bioink printed constructs after printing with different extrusion speed (* $P < 0.05$). g) Live/dead assay of CFs within G-GNR nanocomposite bioink printed constructs under different UV exposure time (* $P < 0.05$). h) Live/dead assay of CFs within different layer of G-GNR nanocomposite bioink printed constructs. i) No significant difference of cell viability was observed between different layers ($P > 0.05$).

$$\eta = K(\delta w / \delta r)^{n-1} \quad (1)$$

where η is the viscosity, K is the consistency, $\delta w / \delta r$ is the shear rate, and n is the power-law exponent. A smaller value of n represents more shear-thinning experienced by the bioink

$$\bar{\tau} = \frac{K}{2} \left(3 + \frac{1}{n} \right)^n \left(\frac{\bar{V}}{R} \right)^n \quad (2)$$

where $\bar{\tau}$ is the mean shear stress, $\bar{V}$ is the flow rate of bioink, and R is the radius of our bioprinter nozzle (100 µm). The results demonstrated that the shear stress of the G-GNR nanocomposite bioink increased linearly from the central axis to the wall of the nozzle and was higher with the increase of the extrusion speed (Figure 4d). As a result, cell viability of above 70% could be achieved when speeds of 5 and 10 µL min⁻¹ were used; however, cell viability decreased at higher speeds (>10 µL min⁻¹), potentially due to the increase of cell death close to the wall of the nozzle exerted by the high shear stresses during the printing process (Figure 4e,f). Therefore, it is

necessary to maintain the extrusion speed below this critical level while using the nanocomposite bioink.

In our photocrosslinking method, surplus free radicals produced by the photoinitiator upon UV induced DNA damage and decreased the cell viability.^[4] Therefore, it is essential to optimize the UV exposure time on the printed constructs. In this study, high cell viabilities were found while using exposure times below 30 s and the prolonged time (>30 s) significantly decreased cell viability ($P < 0.05$) (Figure 4g and Figure S5, Supporting Information). Moreover, we evaluated cell viability of the different layers of a total construct at a fixed 30 s of UV exposure through confocal microscopy. As shown in Figure 4h,i, the top, middle, and bottom layers of the same construct exhibited high viability (>70%) without significant difference between different layers ($P > 0.05$).

2.4. Cellular Growth in the Bioprinted Constructs

To engineer functional cardiac tissue, cardiac cells (CMs:CFs = 1:1, 5×10^6 cells mL⁻¹) harvested from neonatal

rats were coloaded into the G-GNR nanocomposite bioink for printing. After bioprinting, CMs and CFs were found to be homogeneously encapsulated in the microfibers of the constructs (Figure 5a). The 3D cardiac constructs were then cultured in culture medium at 37 °C in a 5% CO₂ humidified incubator. Prior to spreading and proliferation, the encapsulated cells will attach to the cell binding sites on GelMA. Alginate prevents spreading of encapsulated cells, thus reducing their cellular viability as it lacks the necessary cellular binding sites.^[5a] Our previous study found that the crosslinked alginate gel could be dissolved during several days of culture.^[4] We assume that the monovalent potassium ions in culture medium replace the chelated calcium ions from the ionically crosslinked alginate gel.^[24] As a result, the alginate that was ionically crosslinked by calcium ions began to degrade in the cell medium, thus exposing G-GNRs and cell binding sites of the GelMA to the encapsulated cells. After 5 d of culture, good cell spreading was observed in constructs (Figure 5b). On day 12, these cells were found to be elongated and highly confluent within the printed construct that led to the formation of the uniform and interconnected tissue layer (Figure 5c). In addition, to quantitatively evaluate cellular proliferation in printed construct, a presto blue assay was employed to measure the cell growth during the culture period (Figure 5d). Cells encapsulated in the G-GNR nanocomposite and GelMA/alginate bioink-printed constructs exhibited high metabolic activities. However, there were no significant differences in cell proliferation between the G-GNR nanocomposite bioink and pristine GelMA/alginate bioink-printed constructs ($P > 0.05$) (Figure 5d). There was a sharp decrease in the dry weight of the construct during the initial culture period (Figure 5e). We assumed that a substantial amount of uncrosslinked material was lost due to diffusion during the first days of culture. Interestingly, we found that the weight of the construct remained constant from day 14 to day 21. It was speculated that the ECM produced by the cells compensated for the material degradation. During a culture time of 14 d, the global structure of the bioprinted cardiac construct remained intact as well as the inner grid (Figure 5f).

2.5. Function of Cardiac Cells in Printed Construct

Adding G-GNRs to the constructs can create conductive bridges across the matrices, thus connecting adjacent cardiac cell bundles, while promoting electrical propagation, and contributing to the functional improvement of the cells. In our study, G-GNR nanocomposite bioink-printed constructs expressed higher levels of gap junction protein Cx-43 on day 14 than pristine GelMA/alginate bioink-printed ones (Figure 5g,h). Accordingly, we found that the G-GNR nanocomposite bioink-printed constructs exhibited higher synchronized contractile frequency when compared to the pristine GelMA/alginate bioink-printed constructs (Figure 5i and Videos S6 and S7, Supporting Information). This indicated that cellular expression of phenotypic traits was consistent with an increased electrical coupling and contractile properties in the constructs. Another possible underlying reason for the improvement of cardiac cell function in the G-GNR nanocomposite bioink-printed construct is

that the GNRs are ultrafine particles that possess an intrinsic inhibition effect on the overproliferation of CFs by altering the expression of mRNAs that encode for β -actin, α -smooth muscle actin, and collagen type I.^[14a] Although CFs are necessary for cardiac tissue engineering as they participate in essential physiological activities, the highly proliferative CFs in the constructs have the potential to overproliferate and inundate the in vitro construct, thus skewing the initial CM to CF ratio. As a result, there is a pathological simulation of fibrosis that leads to a decrease in the contractile force and an impairment in function. Normally, the CM to CF ratio is tightly regulated and is constantly maintained in healthy individuals.^[12] A previous study introduced gold nanoconstructs into 3D engineered constructs in an effort to achieve improved maintenance of the initial CM to CF ratio while eventually contributing to overall stronger contractile forces.^[9a] In our study, the improved contractile behaviors in the G-GNR nanocomposite constructs could potentially be attributed to the GNRs-based inhibition on excessive CFs proliferation.

The cardiac construct can be efficiently bioprinted and potentially implanted for cardiac repair. One crucial factor that requires further study is the determination of the fate of GNRs in the host system. In most studies, GNRs were systemically administrated and subsequently distributed in organs.^[25] For the implanted cardiac construct, however, it has been speculated that the GNRs could be mainly internalized by the local cardiac cells after the degradation of biomaterial matrix and only a small amount of them may enter circulation system. Previous studies showed an almost immediate clearance of the GNR from the circulation after the administration. In blood serum, GNR immediately forms a protein corona, causing rapid recognition and internalization by components of the reticuloendothelial system and mainly accumulates in liver.^[25] These studies revealed that the accumulated GNRs in a healthy liver did not cause significant hepatotoxicity as evidenced by biochemical and histological measurements, even at high doses (4 $\mu\text{g g}^{-1}$ by weight) of systemically administrated GNR. However, potential harm could be induced in clinically relevant settings of liver injury.^[25b] The dose of GNR in our printed construct was deemed safe; however, particular caution would be required if liver diseases are concomitantly present in clinical scenarios.

3. Conclusion

In conclusion, we have developed and characterized a G-GNR-incorporated nanocomposite bioink for the 3D bioprinting of functional cardiac constructs. We have demonstrated that the presence of G-GNRs in a bioink is both cytocompatible and feasible for printing. Moreover, G-GNRs improved the electrical propagation between cardiac cells and promoted their functional improvement in the printed cardiac construct. We envision that this nanocomposite bioink could be useful in creating a more customized and biomimetic 3D-printed cardiac construct with contractile properties for drug testing, while also having the potential for implantation in an affected area in the heart following myocardial infarction. Furthermore, this technique could also assist in regenerating other electrogenic tissues, such as the spinal cord, brain tissue, and skeletal muscle.

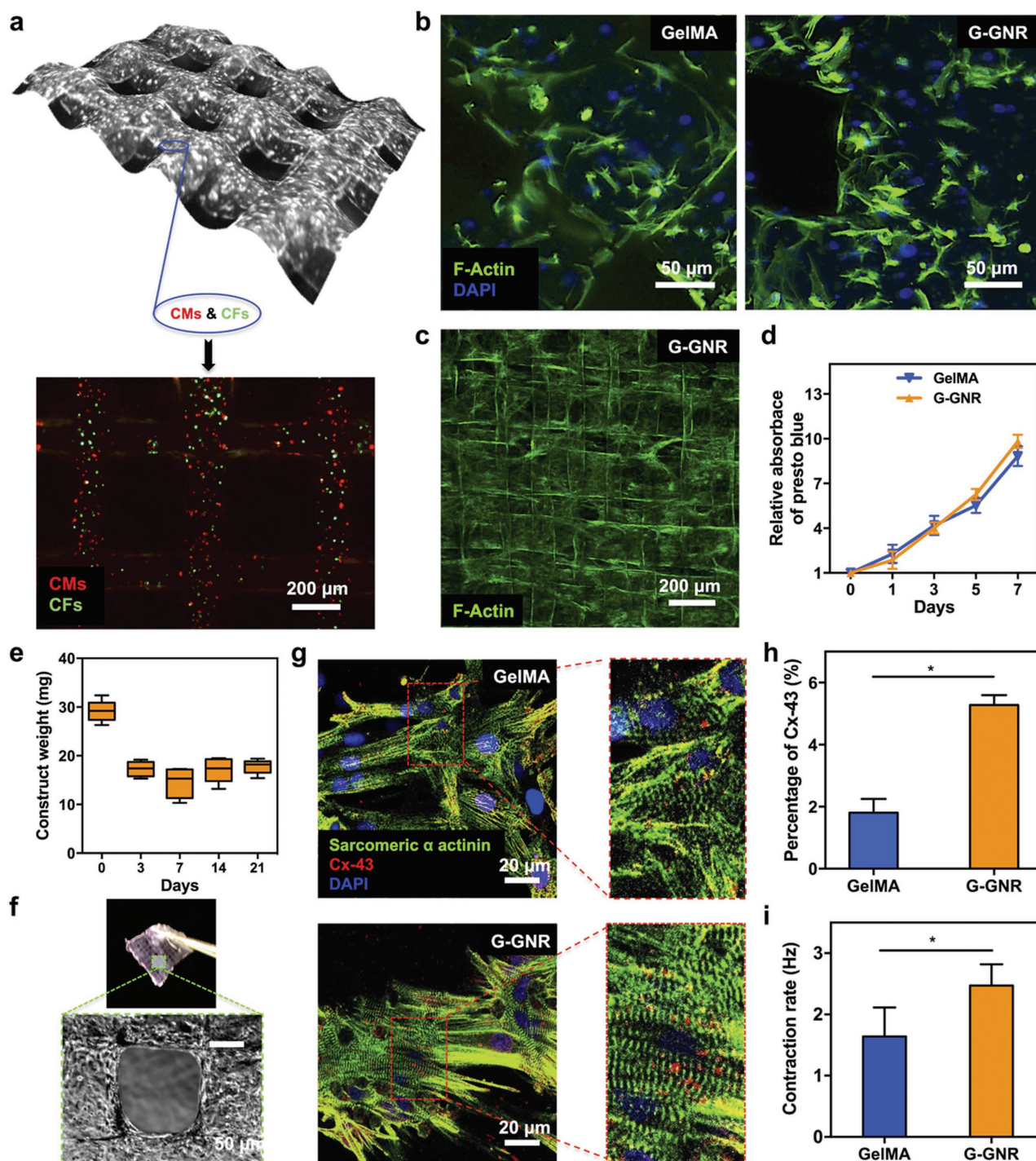


Figure 5. Bioprinted cardiac tissue construct. a) Pseudo-3D bright field image showed cells were homogeneously distributed in bioprinted construct using G-GNR nanocomposite bioink (0.1 mg mL^{-1} G-GNR, 7% GelMA, 2% alginate). Fluorescence cell tracker-labeled CMs (red) and CFs (green) were observed in construct after printing. b) Fluorescence F-actin and DAPI images of bioprinted cardiac cells within GelMA/alginate bioink and G-GNR nanocomposite bioink-printed constructs on day 5. c) Fluorescence F-actin images of bioprinted cardiac cells within G-GNR nanocomposite bioink-printed constructs on day 12. d) Presto blue assay showed that there were no significant differences in cell proliferation within GelMA/alginate bioink and G-GNR nanocomposite bioink-printed constructs. e) The dry weight change of cell-laden G-GNR nanocomposite bioink-printed constructs during culture. f) The morphology and inner grids of the G-GNR nanocomposite bioink-printed construct were maintained during culture. g) Immunostaining of sarcomeric α -actinin (green), nuclei (blue), and Cx-43 (red) revealed that cardiac tissues (14 d culture) within GelMA/alginate bioink and G-GNR nanocomposite bioink-printed constructs were phenotypically different. h) Quantification of Cx-43 expression by the CMs in the printed constructs, plotted as percentages of area coverage calculated from the fluorescence images (* $P < 0.05$). i) Spontaneous beating rates of the GelMA/alginate bioink and G-GNR nanocomposite bioink-printed constructs (* $P < 0.05$).

4. Experimental Section

Materials: GNRs (34 nm long and 25 nm wide), alginate sodium salt from brown algae (low viscosity, 100–300 cP), calcium chloride (CaCl_2), gelatin (type A, 300 Bloom), methacrylic anhydride (MA), and photoinitiator (2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS), fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Dulbecco's modified Eagle medium (DMEM), LIVE/DEAD Viability/Cytotoxicity Kit, PrestoBlue Kit, CellTracker Green 5-chloromethylfluorescein diacetate, CellTracker CM-Dil, Alexa Fluor 488 actin conjugate (F-actin staining), and antibodies (sarcomeric α -actin, connexin-43, troponin I) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

GelMA Synthesis: GelMA was prepared based on our previously reported protocol. In short, type A porcine skin gelatin was dissolved at 60 °C in PBS. Under continuous stirring, MA was added dropwise to the gelatin solution. Unreacted MA was removed through dialysis against deionized water at 50 °C for 7 d. They molecular weight cut off range was 12–14 kDa. Finally, the solution was freeze dried and stored at room temperature for future use.

Cell Isolation and Culture: Following a well-established protocol, which has been approved by the Institute's Committee on Animal Care, neonatal rat ventricular CMs and CFs were isolated. In brief, hearts extracted from 2 d old Sprague–Dawley rats were subjected to series of collagenase treatments. Following a 1 h preplating of isolated cells, CMs and CFs were separated on account of their different attachment time.

Preparation of G-GNRs: GelMA was dissolved in HEPES (7%, w/v) at 50 °C for 10 min. Removal of excess CTAB molecules in C-GNR solution was achieved by triple centrifugation (5 min, 5000 rpm). After the removal of the supernatant, the above GelMA prepolymer solution was added dropwise into the GNRs. G-GNRs were obtained under ultrasonication (VCX 400, 80 W, 2 s on and 1 s off) for 1 h in a water bath. TEM was used to observe the distribution status of G-GNRs. The absorption spectroscopy of G-GNRs (0.1 mg mL⁻¹) was measured using UV–vis spectrophotometer (Synergy 2, Biotek, Winooski, VT, USA). Their zeta potentials were measured on Malvern. In order to obtain G-GNR/GelMA prepolymer solution with GNR target concentrations of 0, 0.1, 0.25, and 0.5 mg mL⁻¹, G-GNRs were added to a 7% GelMA prepolymer solution, containing 0.25% photoinitiator, at various volumes. The pristine 7% GelMA prepolymer solution was used as a control.

Preparation and Evaluation of G-GNR/GelMA Hydrogel: After UV exposure of prepolymer solutions, gels measuring 1 cm × 2.5 cm and at a height of 1 cm were placed in the sample compartment for impedance measurements. A platinum wire was incorporated into the gel to provide electrode contact. Impedance analysis was executed using an electrochemical workstation (CHI660, CH Instruments Inc., Austin, TX, USA). An alternating voltage with amplitude of 0.01 V was applied on the gel, and the impedance values were measured under the frequency from 10 Hz to 100 kHz, respectively. In addition, the mechanical properties of the G-GNR/GelMA hydrogel were tested with an unconfined compressive test. The hydrogel was casted in glass molds (1 mm height), exposed to a UV light source (Omnicure S2000, Excelitas Technologies, Salem, MA, USA) (800 mW) for 30 s to crosslink, and placed in cell culture media inside an incubator to reach the swelling equilibrium. The compressive strength was tested at a cross speed of 30 mm s⁻¹ and a 60% strain level using a mechanical testing machine (Instron Model 5542, Norwood, MA, USA). The slope of the initial linear region, which corresponds to 0%–10% strain, was used to determine the Young's modulus. The porous structure of the G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel was observed under field emission SEM (Ultra55, Carl Zeiss, Oberkochen, Germany). The surface topography of 7% pristine GelMA and G-GNR (0.1 mg mL⁻¹)/GelMA (7%) hydrogel were detected by AFM (Dimension ICON, Bruker Corporation, Billerica, MA, USA). A fluid imaging probe (SCANASYST FLUID+) with a nitride tip on a nitride lever with a tip radius of 0.5–0.7 μm , a resonance frequency of 120–180 kHz and spring constant 0.7 N m⁻¹ was used for measurement. Images were then analyzed using Nanoscope Analysis offline software.

Cell Behaviors in the GNR-Incorporated GelMA Network: The hydrogel film was used to evaluate cellular behavior in the G-GNR-incorporated GelMA network. 10 μL of G-GNR/GelMA prepolymer solution was placed between two 50 μm tall spacers, covered with a 3-(trimethoxysilyl) propyl methacrylate (TMSPMA)-coated glass slide. After exposure to UV light (800 mW), thin films were obtained on TMSPMA-coated glass and cultured in a 12-well plate. To evaluate the cytotoxicity, NIH-3T3 fibroblasts (7.5×10^5 cells per well) were seeded onto C-GNR/GelMA, G-GNR/GelMA, and pristine GelMA hydrogels, respectively. The cell-seeded samples were cultured in DMEM (Gibco, Thermo Fisher scientific, Waltham, MA, USA) containing 10% FBS (Gibco), 1% L-glutamine (Gibco), and 100 units mL⁻¹ penicillin-streptomycin (Gibco). The cell viability was measured using live/dead assay. Briefly, the ethidium homodimer-1 (2 $\mu\text{L mL}^{-1}$) is red in color for the dead cells and calcein acetoxyethyl (AM) (0.5 $\mu\text{L mL}^{-1}$) is green in color for live cells were mixed in PBS to obtain the working solution. After washing the samples, 300 μL of the working solution was added into each sample and incubated at 37 °C for 25 min. Images were observed under fluorescence microscope (Axio Observer D1, Carl Zeiss) and Image J software was used for analysis. Three pictures of different portions of each sample were acquired, using triplicates for each time point.

When CMs (7.5×10^5 cells per well) were seeded onto the hydrogel, their retention, growth, and beating behaviors were observed and recorded using fluorescence microscope equipped with a charge-coupled device (CCD) camera every day. Image J software was used for image analysis. For immunocytochemistry, samples were fixed for 30 min in 4% paraformaldehyde at room temperature. Following fixation, the samples were permeabilized with 0.15% Triton X-100 for 30 min. Thin films were immunostained with F-actin (day 5), sarcomeric α -actinin (day 7), connexin 43 (day 7), and Troponin I (day 7). The nuclei were counterstained with 4'-diamidino-2-phenylindole (DAPI) (1:1000) dilution in PBS. These stained samples were then imaged with a fluorescence microscope.

Bioprinting Using the G-GNR Nanocomposite Bioink: To obtain G-GNR nanocomposite bioink, an alginate prepolymer solution (2%, w/v) was mixed with the G-GNR/GelMA prepolymer solution containing 0.25% photoinitiator. The coaxial extruder was mounted on NovoGen MMX Bioprinter (Organovo, San Diego, CA, USA) to perform the continuous 3D deposition of computer-designed patterns. The internal needle (Diameter = 200 μm) was connected with plastic tubing to a 1 mL syringe containing the bioink, while the external needle (Diameter = 600 μm) was connected to a 1 mL syringe containing the CaCl_2 solution (0.3 M). The deposition speed ranged from 1 to 6 mm s⁻¹ (typically 4 mm s⁻¹). Extrusion speeds were controlled with microfluidic syringe pumps (Harvard Apparatus, Holliston, MA, USA) and usually ranged between 5 and 20 $\mu\text{L min}^{-1}$. The printing procedure was designed to start when both flows reach the tip of the coaxial needles. At the end of the deposition step, the construct was exposed to UV light (800 mW) for a desired amount of time. Constructs were cultured in phenol red-free medium to detect the G-GNR diffusion. From day 1 to day 5, medium was obtained and measured by spectrophotometer from day 1 to day 5. At day 5, the constructs were dissolved in a 1 mg mL⁻¹ collagenase type II solution at 37 °C for a measurement of G-GNRs released from the constructs using spectrophotometer.

To create the gelatin-based supporting bath, the authors mixed 2% gelatin in Milli-Q water containing CaCl_2 (11×10^{-3} M) and dissolved the gelatin at 50 °C for 30 min. After dissolution, the gelatin solution was placed in a chemical hood until the temperature of the gelatin solution returned to room temperature. A mold with dimensions of $1.5 \times 1.5 \times 1.5$ cm³ was filled with the gelatin solution and then placed at 4 °C for 10 min to form a support gel bath. Furthermore, the G-GNR nanocomposite bioink was prepared for embedded printing of the spiral structure. The bioink was extruded through a needle (Diameter = 200 μm), which was mounted on a bioprinter (Organovo) to perform the continuous 3D deposition of the computer-designed patterns. After printing, the printed constructs in the gelatin support gel were crosslinked by UV light (800 mW) for the desired amount of time. Next, the gelatin support gel with the spiral structure was removed

from the mold and placed in PBS at 37 °C. The spiral structure was then obtained from the support gel.

Assessment of Cellular Survival in Bioprinted Constructs: CFs (5×10^6 cells mL⁻¹) were mixed with bioink and stirred mildly at 37 °C to obtain cell-laden bioink. The rheological properties of the G-GNR nanocomposite bioink were analyzed with a rotational rheometer (RHEOPLUS-32, plate-cone geometry, Anton Paar, Ashland, VA, USA). Bioink without G-GNRs was named as GelMA/alginate bioink and used as a control. In all test samples, the concentration of GelMA was set as 7%. Samples were kept under mild stirring at an ambient temperature for ≈ 2 h before acquisition. The test was performed in a small closed chamber at 25 °C to prevent evaporation. Two loading cycles, with 10 min interval, were performed before acquiring the rheological data. The velocity distribution of the bioink is analyzed in the needle's nozzle using computational fluid dynamics. The computational geometry of the needle is an axisymmetric tube with the radius of 100 μ m. A flat flow velocity profile was used at the inlet and the extrusion speed was set at 10 μ L min⁻¹. The viscosity was based on the experimental results. The coupling between the pressure and velocity field was determined using the SIMPLE algorithm with second-order upwind scheme for the momentum equations. The convergence was believed to be obtained when the residuals of continuity and momentum were below 10^{-6} .^[3] Using a validated finite volume-based algorithm Fluent (Ansys, Inc., Concord, MA, USA), the numerical calculations were carried out. The cell viability in the bioprinted constructs under different extrusion speed and different UV times were measured by live/dead assay based on the manufacturer's protocol on day 1. The cell viability of different sections of stacked constructs was also measured on day 1 using a confocal microscope.

Assessment of Cellular Growth in Bioprinted Constructs: To print cardiac constructs, CMs and CFs (1:1, 5×10^6 cells mL⁻¹) were coloaded into G-GNR nanocomposite bioink and GelMA/alginate bioink, respectively. To visualize the cells printed on the constructs, images under an optical microscope were analyzed using custom-coded MATLAB programs. Also, fluorescent cell trackers were used to label CMs (red) and CFs (green) before bioink preparation. At day 1 and 3, the cell viability within the constructs was determined by performing a live/dead assay based on the manufacturer's protocol. From 0 to 7 d after printing, cell growth in constructs was evaluated by PrestoBlue kit and measured using spectrophotometry. At day 5 and day 12, immunostaining of F-actin were conducted on constructs and observed under a laser scanning confocal microscope (SP5X MP, Leica, Wetzlar, Germany). Another batch of constructs were lyophilized and weighed for degradation tests.

Assessment of Cellular Function in Bioprinted Constructs: CM beating was observed using an optical microscope and analyzed using custom-coded MATLAB programs. At day 14, immunostaining of sarcomeric α -actinin and Cx-43 was conducted on constructs and observed under a laser scanning confocal microscope. The percentage of Cx-43 was quantified using Image J.

Statistics: Using SPSS 17.0 software (SPSS, Chicago, IL, USA) the data were analyzed, and all values were expressed as the mean $\pm$ the standard error. The significant difference among multiple groups was assessed using one-way analysis of variance with the post hoc Bonferroni test. Between two groups, the significant difference was evaluated by the Student's *t*-test. All differences were statistically significant when $P < 0.05$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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- [1] a) S. V. Murphy, A. Atala, *Nat. Biotechnol.* **2014**, *32*, 773; b) I. T. Ozbolata, M. Hospodiuk, *Biomaterials* **2016**, *76*, 321; c) H. W. Kang, S. J. Lee, I. K. Ko, C. Kengla, J. J. Yoo, A. Atala, *Nat. Biotechnol.* **2016**, *34*, 312.
- [2] D. B. Kolesky, R. L. Truby, A. S. Gladman, T. A. Busbee, K. A. Homan, J. A. Lewis, *Adv. Mater.* **2014**, *26*, 3124.
- [3] A. Blaeser, D. F. D. Campos, U. Puster, W. Richtering, M. M. Stevens, H. Fischer, *Adv. Healthcare Mater.* **2015**, *5*, 326.
- [4] C. Colosi, S. R. Shin, V. Manoharan, S. Massa, M. Costantini, A. Barbetta, M. R. Dokmeci, M. Dentini, A. Khademhosseini, *Adv. Mater.* **2015**, *28*, 677.
- [5] a) A. Tamayol, A. H. Najafabadi, B. Aliakbarian, E. Arab-Tehrany, M. Akbari, N. Annabi, D. Juncker, A. Khademhosseini, *Adv. Healthcare Mater.* **2015**, *4*, 2146; b) W. Jia, P. S. Gungor-Ozkerim, Y. S. Zhang, K. Yue, K. Zhu, W. Liu, Q. Pi, B. Byambaa, M. R. Dokmeci, S. R. Shin, A. Khademhosseini, *Biomaterials* **2016**, *106*, 58.
- [6] K. Yue, G. T. Santiago, M. M. Alvarez, A. Tamayol, N. Annabi, A. Khademhosseini, *Biomaterials* **2015**, *73*, 254.
- [7] a) N. Annabi, S. R. Shin, A. Tamayol, M. Miscuglio, M. A. Bakooshli, A. Assmann, P. Mostafalu, J. Y. Sun, S. Mithieux, L. Cheung, X. S. Tang, A. S. Weiss, A. Khademhosseini, *Adv. Mater.* **2016**, *28*, 40; b) S. R. Shin, S. M. Jung, M. Zalabany, K. Kim, P. Zorlutuna, S. B. Kim, M. Nikkhah, M. Khabiry, M. Azize, J. Kong, K. T. Wan, T. Palacios, M. R. Dokmeci, H. Bae, X. S. Tang, A. Khademhosseini, *ACS Nano* **2013**, *7*, 2369; c) T. Dvir, B. P. Timko, M. D. Bringham, S. R. Naik, S. S. Karajanagi, O. Levy, H. Jin, K. K. Parker, R. Langer, D. S. Kohane, *Nat. Nanotechnol.* **2011**, *6*, 720.
- [8] a) C. M. Cobley, J. Chen, E. C. Cho, L. V. Wang, Y. Xia, *Chem. Soc. Rev.* **2011**, *40*, 44; b) L. Vigderman, B. P. Khanal, E. R. Zubarev, *Adv. Mater.* **2012**, *24*, 4811.
- [9] a) M. Shevach, S. Fleischer, A. Shapira, T. Dvir, *Nano Lett.* **2014**, *14*, 5792; b) S. Fleischer, M. Shevach, R. Feinerab, T. Dvir, *Nanoscale* **2014**, *6*, 9410; c) A. Navaei, H. Saini, W. Christenson, R. T. Sullivan, R. Ros, M. Nikkhah, *Acta Biomater.* **2016**, *41*, 133.
- [10] F. Pati, J. Jang, D. H. Ha, S. W. Kim, J. W. Rhee, J. H. Shim, D. H. Kim, D. W. Cho, *Nat. Commun.* **2014**, *5*, 3935.

- [11] I. Banerjee, J. W. Fuseler, R. L. Price, T. K. Borg, T. A. Baudino, *Am. J. Physiol.: Heart Circ. Physiol.* **2007**, 293, H1883.
- [12] J. E. Cartledge, C. Kane, P. Dias, M. Tesfom, L. Clarke, B. Mckee, S. A. Ayoubi, A. Chester, M. H. Yacoub, P. Camelliti, C. M. Terracciano, *Cardiovasc. Res.* **2015**, 105, 260.
- [13] H. Saini, A. Navaei, A. V. Putten, M. Nikkhah, *Adv. Healthcare Mater.* **2015**, 4, 1961.
- [14] a) P. N. Sisco, C. G. Wilson, E. Mironova, S. C. Baxter, C. J. Murphy, E. C. Goldsmith, *Nano Lett.* **2008**, 8, 3409; b) C. G. Wilson, P. N. Sisco, F. A. Gadala-Maria, C. J. Murphy, E. C. Goldsmith, *Biomaterials* **2009**, 30, 5639.
- [15] L. Wang, X. Jiang, Y. Ji, R. Bai, Y. Zhao, X. Wu, C. Chen, *Nanoscale* **2013**, 5, 8384.
- [16] A. Indrasekara, R. C. Wadams, L. Fabris, *Part. Part. Syst. Character.* **2014**, 31, 819.
- [17] a) Y. Qiu, Y. Liu, L. Wang, L. Xu, R. Bai, Y. Ji, X. Wu, Y. Zhao, Y. Li, C. Chen, *Biomaterials* **2010**, 31, 7606; b) T. S. Hauck, A. A. Ghazani, W. C. Chan, *Small* **2008**, 4, 153; c) J.-H. Wang, B. Wang, Q. Liu, Q. Li, H. Huang, L. Song, T.-Y. Sun, H. Wang, X.-F. Yu, C. Li, *Biomaterials* **2013**, 34, 4274.
- [18] J. Zhang, M. Gu, T. Zheng, J. Zhu, *Anal. Chem.* **2009**, 81, 6641.
- [19] L. R. Madden, D. J. Mortisen, E. M. Sussman, S. K. Dupras, J. A. Fugate, J. L. Cuy, K. D. Hauch, M. A. Laflamme, C. E. Murry, B. D. Ratner, *Proc. Natl. Acad. Sci. USA* **2010**, 107, 15211.
- [20] B. Bhana, R. K. Iyer, W. L. K. Chen, R. Zhao, K. L. Sider, M. Likhitanichkul, C. A. Simmons, M. Radisic, *Biotechnol. Bioeng.* **2010**, 105, 1148.
- [21] N. Hersch, B. Wolters, G. Dreissen, R. Springer, N. Kirchgeßner, R. Merkel, B. Hoffmann, *Biol. Open* **2013**, 2, 351.
- [22] T. J. Hinton, Q. Jallerat, R. N. Palchesko, J. H. Park, M. S. Grodzicki, H. J. Shue, M. H. Ramadan, A. R. Hudson, A. W. Feinberg, *Sci. Adv.* **2015**, 1, e1500758.
- [23] R. D. Farahani, M. Dubé, D. Therriault, *Adv. Mater.* **2016**, 28, 5794.
- [24] S. H. Hong, M. Shin, J. Lee, J. H. Ryu, S. Lee, J. W. Yang, W. D. Kim, H. Lee, *Adv. Healthcare Mater.* **2015**, 5, 75.
- [25] a) R. Fernandez-Ruiz, M. J. Redrejo, E. J. Friedrich, M. Ramos, T. Fernandez, *Anal. Chem.* **2014**, 86, 7383; b) M. Bartneck, T. Ritz, H. A. Keul, M. Wambach, J. Bornemann, U. Gbureck, J. Ehling, T. Lammers, F. Heymann, N. Gassler, *ACS Nano* **2012**, 6, 8767.